From Skeleton to Cytoskeleton

Osteocalcin Transforms Vascular Fibroblasts to Myofibroblasts Via Angiotensin II and Toll-Like Receptor 4

Chi Yung Yuen,* Siu Ling Wong,* Chi Wai Lau,* Suk-Ying Tsang, Aimin Xu, Zhiming Zhu, Chi Fai Ng, Xiaoqiang Yao, Siu Kai Kong, Hung Kay Lee, Yu Huang

Rationale: **The expression of osteocalcin is augmented in human atherosclerotic lesions. How osteocalcin triggers vascular pathogenesis and remodeling is unclear.**

Objective: **To investigate whether osteocalcin promotes transformation of adventitial fibroblast to myofibroblasts and the molecular mechanism involved.**

Methods and Results: **Immunohistochemistry indicated that osteocalcin was expressed in the neointima of renal arteries from diabetic patients. Western blotting and wound-healing assay showed that osteocalcin induced fibroblast transformation and migration, which were attenuated by blockers of the renin-angiotensin system and protein kinase Cδ (PKCδ), toll-like receptor 4 (TLR4) neutralizing antibody, and antagonist and inhibitors of free radical production and cyclooxygenase-2. Small interfering RNA silencing of TLR4 and PKCδ abolished fibroblast transformation. Angiotensin II level in the conditioned medium from the osteocalcin-treated fibroblasts was found elevated using enzyme immunoassay. Culturing of fibroblasts in conditioned medium collected from differentiated osteoblasts promoted fibroblast transformation. The expression of fibronectin, TLR4, and cyclooxygenase-2 is augmented in human mesenteric arteries after 5-day in vitro exposure to osteocalcin.**

Conclusions: **Osteocalcin transforms adventitial fibroblasts to myofibroblasts through stimulating angiotensin II release and subsequent activation of PKCδ/TLR4/reactive oxygen species/cyclooxygenase-2 signaling cascade. This study reveals that the skeletal hormone osteocalcin cross-talks with vascular system and contributes to vascular remodeling. (*Circ Res*. 2012;111:e55-e66.)**

Key Words: fibroblast transformation ■ osteocalcin ■ angiotensin II ■ protein kinase C ■ toll-like receptor 4

Vascular dysfunction has traditionally been considered an "inside-out" process in blood vessels,[1] in which vascular inflammation is initiated at the intimal surface of the artery as a consequence of overproduction of inflammatory mediators such as reactive oxygen species (ROS) in the endothelium. Until recently, growing evidence has suggested an "outside-in" hypothesis,[1] proposing that inflammation may originate from the adventitial fibroblasts and then progress toward the intima, culminating in vascular wall thickening and dysfunction. Neointima formation is partially characterized by the acquisition of migratory and proliferative ability of fibroblasts after their transformation to myofibroblasts, signified by the induced expression of cytoskeletal proteins such as α-smooth muscle actin (α-SMA) and the excessive synthesis and secretion of matrix components including fibronectin.[2] These phenotypic alterations allow adventitial fibroblasts to migrate toward the lumen, contributing to neointima formation and intimal thickening.[2,3] The subsequent narrowing of arterial lumen is one of the hallmark features of vascular remodeling. Adventitial fibroblasts can differentiate to myofibroblasts in response to vascular injury, as revealed by their localization in the neointima of the balloon-induced intimal lesions.[4–6] Importantly, myofibroblasts further promote the expression of growth factors and inflammatory cytokines

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Non-standard Abbreviations and Acronyms	
α-SMA	α-smooth muscle actin
Ang II	angiotensin II
AT_1R	angiotensin II type 1 receptor
COX-2	cyclooxygenase-2
LPS	lipopolysaccharide
PKCδ	protein kinase Cδ
RAS	renin-angiotensin system
ROS	reactive oxygen species
SMC	smooth muscle cell
TLR4	toll-like receptor 4
VSMC	vascular smooth muscle cell

such as transforming growth factor β-1,[7] which are involved in the conversion of adventitial fibroblasts to myofibroblasts,[8] thereby promoting neointima development. Collectively, these findings underscore a close link in the inflammatory response between fibroblasts and myofibroblast formation.

The pathogenesis of vascular dysfunction is associated with chronic inflammation[1] in which the immune system plays a pivotal role in its initiation and progression.[9] Toll-like receptor 4 (TLR4), a type I transmembrane receptor that participates in innate immunity, is implicated in arterial remodeling through activation of certain proinflammatory factors in the vascular wall.[10] TLR4 is expressed in human and murine arterial lesions,[11] and its upregulation in intimal hyperplasia suggests its positive contribution to the development of atherosclerosis.[12] Deletion of TLR4 gene attenuates the development of atherosclerosis in apoE-deficient mice.[13] These results unambiguously imply that TLR4 serves as a significant inflammatory mediator in the pathogenesis of atherosclerosis.

Growing evidence points to an interplay between bone pathology and cardiovascular diseases.[14,15] For example, the formation of human atherosclerotic plaques resembles developmental osteogenesis, evidenced by the production of bone-associated proteins.[16–18] The noncollagenous bone matrix protein, osteocalcin, represents the most abundant molecule synthesized by osteoblasts during the course of bone remodeling and is implicated in atherosclerosis and vascular remodeling.[19] Consistently, elevated expression of osteocalcin is detected in atherosclerotic plaques and arteries.[20] Moreover, a significantly higher serum level of osteocalcin is reported in patients with atherosclerosis[21] and it also correlates with an increased prevalence of carotid atherosclerosis among postmenopausal women.[22] Endothelial progenitor cells in patients with coronary atherosclerosis exhibit an elevated expression of osteocalcin,[23] and retention of such osteocalcin-expressing endothelial progenitor cells in arteries results in endothelial dysfunction.[24] Although recent experimental and clinical data have both unequivocally indicated an intimate link between osteocalcin and atherosclerosis, the exact molecular mechanisms remain vastly uncharacterized.

The present experiments were designed to test the hypothesis that osteocalcin promotes transformation of adventitial fibroblast to myofibroblasts depending on TLR4. We demonstrate that osteocalcin triggers adventitial fibroblasts to produce and release angiotensin II (Ang II) and in an autocrine manner, the latter activates the protein kinase Cδ (PKCδ)/TLR4/cyclooxygenase-2 (COX-2) inflammatory cascade to mediate its transformation into myofibroblasts. This study provides novel evidence showing a pathogenic linkage between the skeletal hormone osteocalcin to vascular remodeling through increased synthesis of the cytoskeleton α-SMA in adventitial fibroblasts.

Methods

The animal study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by the Experimental Animal Ethics Committee, Chinese University of Hong Kong. Human renal and mesenteric arteries were obtained after informed consent from patients undergoing nephrectomy or intestinal resection. The use of human arteries for research purposes was approved by the Joint Chinese University of Hong Kong-New Territories East Cluster Clinical Research Ethics Committee.

Expression of osteocalcin and/or other proinflammatory/remodeling markers was detected in human renal arteries and osteocalcin-treated human mesenteric arteries using immunohistochemistry. Mechanistic studies were performed on primary culture of rat aortic adventitial fibroblasts unless otherwise specified. Flow cytometry, tissue culture of human mesenteric arteries, cell culture of fibroblasts and osteoblasts, transfer experiments of conditioned medium, siRNA silencing of PKCδ and TLR4, Western blot analysis, immunohistochemistry, immunofluorescence microscopy, enzyme immunoassay for Ang II and osteocalcin, dihydroethidium (DHE) fluorescence microscopy and electron paramagnetic resonance on ROS production, and wound-healing assay were used in the present study. Data were analyzed by Student *t* test or 1-way ANOVA followed by the Bonferroni post hoc test using Graphpad Prism Software. An expanded Methods section is available in the Online Data Supplement.

Results

Inflammatory Markers and Remodeling Proteins in Human Neointima

Neointima of renal arteries from diabetic patients exhibited positive immunohistochemical staining of the skeletal hormone osteocalcin, proinflammatory markers TLR4 and COX-2, remodeling protein α-SMA, and fibroblast marker vimentin (Figure 1A). By contrast, neointima was not observed in the renal arteries from nondiabetic subjects (Figure 1A). Medium harvested from 5-day incubation of mesenteric arteries of normotensive subjects in osteocalcin in vitro showed a markedly elevated Ang II production, which was prevented by captopril (100 nmol/L), an angiotensin-converting enzyme (ACE) inhibitor (Figure 1B, left panel). Immunohistochemistry showed a positive staining of Ang II across the vascular wall of the osteocalcin-treated human mesenteric arteries, in which smooth muscle was most strongly stained, in addition to the neointima and the adventitial layer (Figure 1B, right), suggesting the possibility for Ang II to act in a paracrine/autocrine manner across the vascular wall. The presence of these markers is indicative of an inflammatory status in the neointima. The expression of vimentin implies the possible participation of adventitial fibroblasts in vascular remodeling while coexistence of vi-

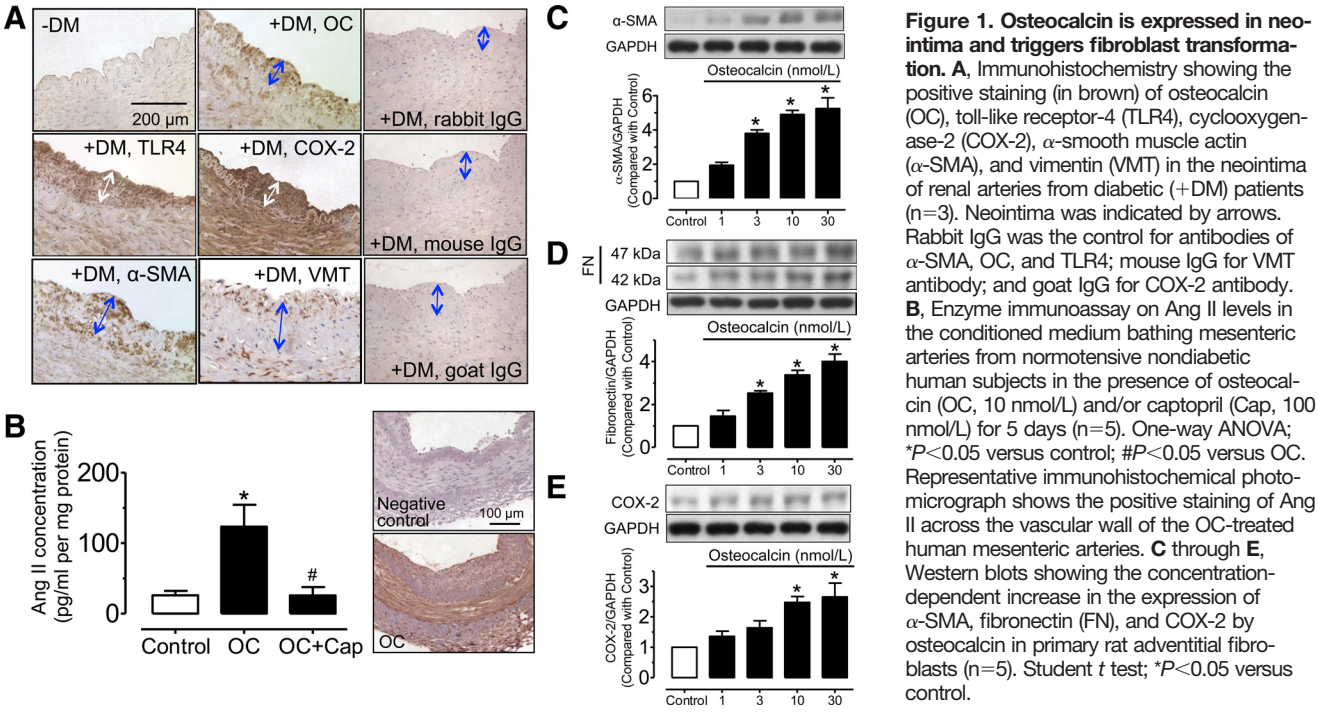

Figure 1. Osteocalcin is expressed in neointima and triggers fibroblast transformation. A, Immunohistochemistry showing the positive staining (in brown) of osteocalcin (OC), toll-like receptor-4 (TLR4), cyclooxygenase-2 (COX-2), α-smooth muscle actin (α-SMA), and vimentin (VMT) in the neointima of renal arteries from diabetic (+DM) patients (n=3). Neointima was indicated by arrows. Rabbit IgG was the control for antibodies of α-SMA, OC, and TLR4; mouse IgG for VMT antibody; and goat IgG for COX-2 antibody. **B**, Enzyme immunoassay on Ang II levels in the conditioned medium bathing mesenteric arteries from normotensive nondiabetic human subjects in the presence of osteocalcin (OC, 10 nmol/L) and/or captopril (Cap, 100 nmol/L) for 5 days (n=5). One-way ANOVA; *$P<0.05$ versus control; #$P<0.05$ versus OC. Representative immunohistochemical photomicrograph shows the positive staining of Ang II across the vascular wall of the OC-treated human mesenteric arteries. **C** through **E**, Western blots showing the concentration-dependent increase in the expression of α-SMA, fibronectin (FN), and COX-2 by osteocalcin in primary rat adventitial fibroblasts (n=5). Student *t* test; *$P<0.05$ versus control.

mentin and α-SMA suggests the transformation of adventitial fibroblasts to myofibroblasts.

Osteocalcin Induces Transformation of Adventitial Fibroblasts

We next tested the effect of osteocalcin to establish its positive role in fibroblast transformation into myofibroblasts. To confirm the identity and purity of the primary culture of cells from the rat aortic adventitia, we first performed Western blot analysis, which showed that the cells were positive to the fibroblast marker prolyl 4-hydroxylase (P4H) while negative to endothelial cell marker PECAM-1 and smooth muscle cell marker α-SMA (Online Figure IA). Second, FACS analysis also showed that the primary culture of cells is 94.3% and 96.1% positive to the fibroblast markers, vimentin and P4H,[25–27] respectively, of the gated population M1. Only <0.3% of cells are positive to PECAM-1, <2% to α-SMA, and <0.4% to myeloid cell marker CD33 (Online Figure IB). These data suggest that the primary culture was likely composed of fibroblasts homogeneously. Twenty-four-hour treatment with osteocalcin (1–30 nmol/L) concentration-dependently stimulated the expression of α-SMA and fibronectin (Figure 1C and 1D) in the fibroblasts, indicating a causal role of osteocalcin in inducing fibroblast transformation. The myofibroblastic phenotype was evidenced by the formation of myofiber as observed under high power fluorescence microscopy (Online Figure IIA) and the focal contraction of the osteocalcin-treated fibroblasts (Online Videos I and II). The expression of vascular remodeling markers was accompanied by a concentration-dependent upregulation of the COX-2 expression by osteocalcin (Figure 1E).

Although we cannot provide direct staining evidence showing the participation of the adventitia through immunohistochemistry, because the adventitia is rich in various matrix proteins and fibers that pick up nonspecific staining readily, we performed experiments to support the role of adventitia in the expression of the remodeling markers. Instead of isolating the fibroblasts, the adventitia of the rat aorta was directly subjected to osteocalcin treatment. The purpose was to test whether the similar observation of the remodeling-induction effect of osteocalcin could be also obtained in the whole adventitia at the tissue level without enzymatic digestion for individual fibroblasts. Indeed, the adventitia was positive to fibroblast markers vimentin and P4H and negative in α-SMA (in untreated control), indicating that the adventitia isolation was clean without much contamination from the smooth muscle layer, which would otherwise give a strong signal for α-SMA. After 24-hour osteocalcin treatment, α-SMA and fibronectin were significantly upregulated as those observed in primary culture of fibroblasts (Online Figure IIB), showing the positive involvement of adventitia in remodeling directly.

Osteocalcin-Induced Fibroblast Transformation Is Mediated by Ang II, TLR4, and COX-2

Captopril (100 nmol/L) and 2 distinct Ang II type 1 receptor (AT_1R) blockers, losartan (3 μmol/L) and ZD 7155 (3 μmol/L), attenuated or abolished osteocalcin (10 nmol/L)-stimulated expressions of α-SMA, fibronectin, COX-2, and TLR4 (Figure 2A and Online Figure IIIA through D). Ang II (100 nmol/L), as a positive control, also induced the expression of these 4 proteins, which was prevented by losartan (Figure 2A and Online Figure IIIA through D). Together with the expression of the components of renin-angiotensin system (RAS) such as ACE and AT_1R in the fibroblasts (Online Figure IIC) and the upregulation of renin level in the osteocalcin-stimulated fibroblasts (Online Figure IID), the present results indicate the participation and triggering role of the RAS in fibroblast transformation.

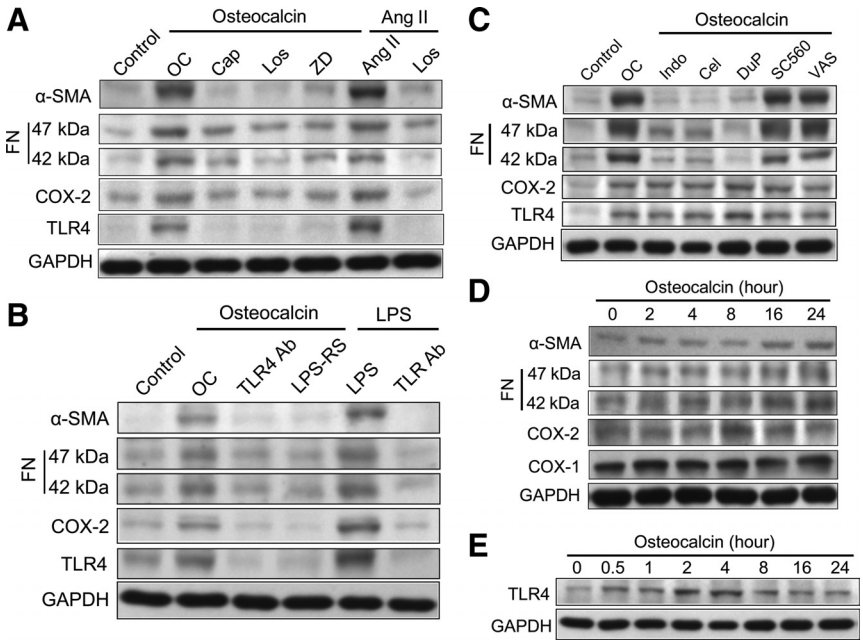

Figure 2. Ang II, TLR4, and COX-2 mediate osteocalcin-induced fibroblast transformation. A through **C**, Western blots showing the effect of **A**, RAS inhibitors (Cap, captopril; ACE inhibitor, 100 nmol/L; Los, losartan; AT_1R antagonist, 3 μmol/L; ZD, ZD 7155; AT_1R antagonist, 3 μmol/L) (n=5–7); **B**, TLR4 neutralizing antibody (TLR4 Ab, 1 μg/mL), TLR4 receptor antagonist (LPS-RS, 10 ng/mL) (n=5–7); and **C**, COX inhibitors (Indo, indomethacin; nonspecific COX inhibitor, 3 μmol/L; Cel, celecoxib; COX-2 inhibitor, 3 μmol/L; DuP, DuP-697; COX-2 inhibitor, 3 μmol/L; SC 560, COX-1 inhibitor, 10 nmol/L; VAS, valeryl salicylate, COX-1 inhibitor, 30 μmol/L) in osteocalcin-induced expression of α-SMA, FN, COX-2, and TLR4 (n=5–7). **D** through **E**, Time course study of the expression levels of **D**, α-SMA, FN, COX-2, and COX-1, and **E**, TLR4 in response to osteocalcin (10 nmol/L) (n=5).

TLR4 neutralizing antibody (1 μg/mL) and a TLR4 antagonist, lipopolysaccharide (LPS)-RS (10 ng/mL), abolished the osteocalcin-induced expression of α-SMA, fibronectin, COX-2, and TLR4 (Figure 2B and Online Figure IIIE through H). Likewise, the TLR4 agonist LPS (10 μg/mL)[28] also upregulated α-SMA, fibronectin, COX-2, and TLR4, and these effects were again inhibited by TLR4 neutralizing antibody (Figure 2B and Online Figure IIIE through H), suggesting that TLR4 not only mediates fibroblast transformation but also modulates the expression of COX-2 and TLR4 per se.

Osteocalcin-triggered upregulation of α-SMA and fibronectin was curtailed by a nonselective COX inhibitor, indomethacin (3 μmol/L), and 2 specific COX-2 inhibitors, celecoxib (3 μmol/L) and DuP 697 (3 μmol/L) (Figure 2C and Online Figure III-I and J), but was insensitive to COX-1 inhibitors, SC560 (10 nmol/L), and valeryl salicylate (VAS, 30 μmol/L) (Figure 2C and Online Figure III-I and J), indicating that osteocalcin-induced α-SMA and fibronectin expression is COX-2-dependent. Unlike TLR4, celecoxib and DuP 697 did not alter the osteocalcin-induced COX-2 expression (Figure 2C and Online Figure IIIK), suggesting that COX-2–derived products did not regulate COX-2 expression. Osteocalcin-stimulated TLR4 expression was not changed by COX-2 inhibitors, suggesting that TLR4 is likely to act upstream of COX-2 in the signaling cascade (Figure 2C and Online Figure IIIL).

Osteocalcin (10 nmol/L) increased the expression of α-SMA, fibronectin, TLR4, and COX-2 in a time-dependent manner. While the maximum expression of α-SMA and fibronectin occurred at 24 hours after osteocalcin exposure (Figure 2D and Online Figure IVA and B), the expression of TLR4 and COX-2 peaked at 2 and 8 hours, respectively (Figure 2D, 2E, and Online Figure IVC and D). COX-1 expression was unaffected by osteocalcin (Figure 2D and Online Figure IVE). Taken in conjunction with the experiments using pharmacological inhibitors, our findings suggest a sequential activation of TLR4, followed by COX-2 upregulation leading to the induction of α-SMA and fibronectin.

PKCδ Mediates Osteocalcin-Induced COX-2 Expression

Because PKCδ can regulate COX-2 expression in various cell types including endothelial cells,[29] we further examined whether PKC was involved in osteocalcin-induced COX-2-mediated fibroblast transformation. Osteocalcin-induced expression of α-SMA and fibronectin was attenuated by a broad-spectrum PKC inhibitor, GF 109203X (2 μmol/L), and by the specific PKCδ inhibitor rottlerin (10 μmol/L). By contrast, Go 6976 (1 μmol/L, PKCα/β inhibitor) and εV1–2 (10 μmol/L, PKCε inhibitor) had no effect (Figure 3A and 3B). The role of PKC was strengthened by a similar induction of the remodeling markers in response to an exogenous PKC activator, phorbol 12-myristate 13-acetate (PMA), and the effect of PMA was prevented by rottlerin (Figure 3A and 3B). Likewise, the osteocalcin-stimulated expression of TLR4 and COX-2 was attenuated by GF 109203X and rottlerin (Figure 3C and 3D). PMA also stimulated the rottlerin-sensitive expression of TLR4 and COX-2 (Figure 3C and 3D), indicating that PKCδ is upstream of not only COX-2 but also TLR4. Osteocalcin time-dependently activated PKCδ at Thr505, with peak phosphorylation at 4 minutes (Figure 3E). Osteocalcin- and PMA-stimulated PKCδ phosphorylation was reversed by rottlerin (Figure 3F).

To further confirm the essential intermediary role of PKCδ and its position relative to TLR4 in the signaling cascade, small interfering RNA (siRNA) targeting TLR4 (siTLR4) and PKCδ (siPKCδ) was transfected into adventitial fibroblasts. Unlike the nontransfected control or cells transfected with scramble siRNA, the transfected cells did not exhibit an induced expression of α-SMA and fibronectin in response to osteocalcin (Figure 3G and 3H). Successful knock-down of TLR4 and PKCδ was verified by the very minimal expression of both proteins in the respective transfected cells (Figure 3I).

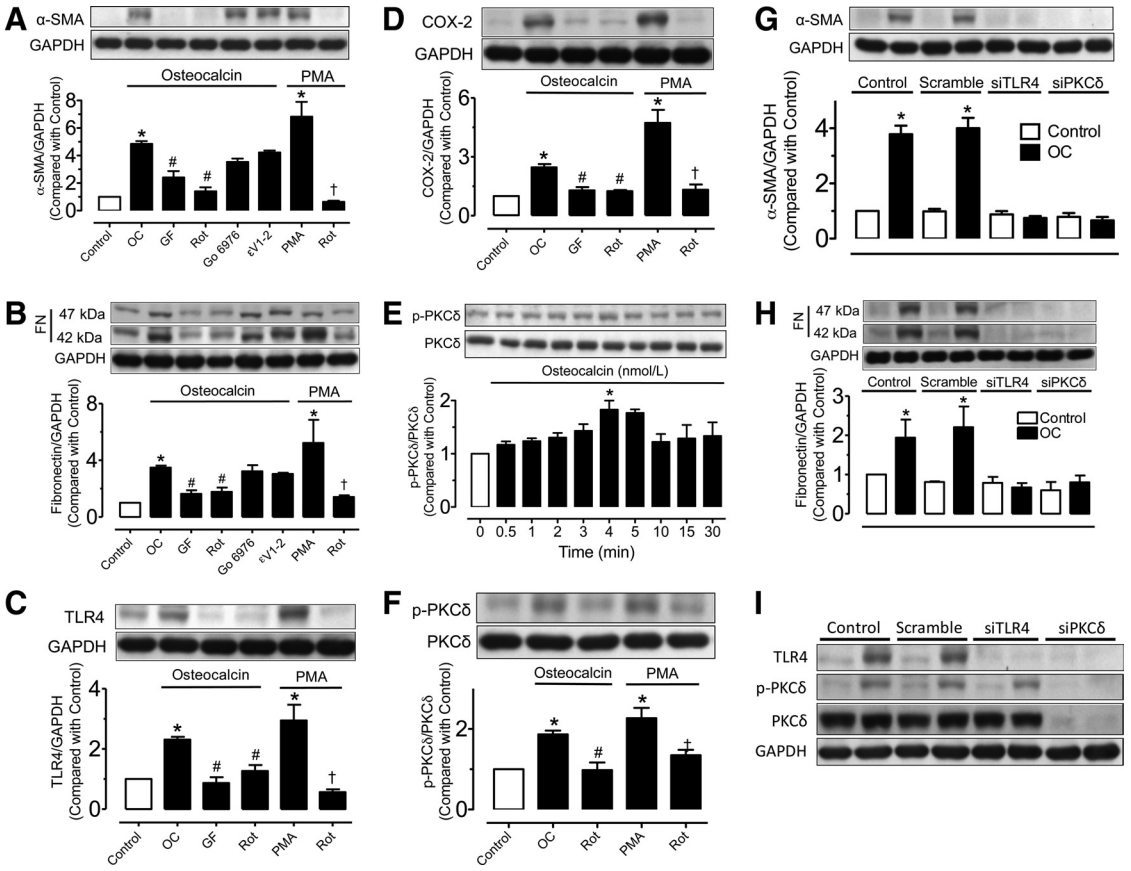

Figure 3. PKCδ mediates osteocalcin-induced fibroblast transformation. A through **D**, Western blots showing the effects of a broad-spectrum PKC inhibitor, GF 109203X (GF, 2 μmol/L), and specific inhibitors against PKCα/β, PKCδ and PKCε, respectively Go6976 (Go, 1 μmol/L), rottlerin (Rot, 10 μmol/L), and εV1–2 (10 μmol/L), on the osteocalcin-induced expression of **A**, α-SMA; **B**, FN; **C**, TLR4; and **D**, COX-2 (n=5–6). **E**, Time-course study of the phosphorylation of PKCδ at the activation site Thr 505 (n=5). **F**, Effect of rottlerin (Rot) on osteocalcin- or PMA-induced PKCδ phosphorylation (n=5). **G** and **H**, Effects of small interfering RNA targeting TLR4 (siTLR4) or PKCδ (siPKCδ) on the osteocalcin-induced expression of **G**, α-SMA, and **H**, FN (n=5). **I**, Confirmation of TLR4 and PKCδ knock-down by siTLR4 and siPKCδ (n=5). One-way ANOVA; *$P<0.05$ versus control; #$P<0.05$ versus OC; †$P<0.05$ versus PMA.

Notably, the TLR4 expression was abolished in fibroblasts transfected with siPKCδ, whereas the PKCδ expression was unaffected in siTLR4-transfected cells, again supporting the regulation of TLR4 by PKCδ and the activation sequence of PKCδ to TLR4.

Osteocalcin Stimulates Production of ROS

DHE fluorescence visualized under confocal microscopy showed that osteocalcin increased the ROS production, and this increase was reversed by captopril, losartan, LPS-RS, and rottlerin but not celecoxib (Figure 4A and 4B), suggesting that ROS is positioned downstream of PKCδ but upstream of COX-2. Intermediate triggers such as Ang II, LPS, and PMA also stimulated ROS increase (Figure 4A and 4B). Western blot analysis showed that osteocalcin-induced expression of α-SMA, fibronectin, and COX-2 was attenuated or abolished by tempol (100 μmol/L, ROS scavenger) and diphenyleneiodonium chloride (DPI, putative NADPH oxidase inhibitor, 100 nmol/L) (Figure 4C through 4E). The lack of effect of tempol and DPI on the TLR4 expression indicates that TLR4 expression is not regulated by the ROS generated downstream (Figure 4F).

Osteocalcin-stimulated ROS production was confirmed with electron paramagnetic resonance. Using TEMPONE-H as the spin-trapping agent, the electron paramagnetic resonance results showed that osteocalcin (10 nmol/L) significantly elevated intracellular ROS level, which is prevented by pretreatments with captopril or LPS-RS (Online Figure VA and C). Ang II (100 nmol/L) also stimulated ROS production, and the effect was abolished by DPI (Online Figure VB and C).

Conditioned Medium Triggers Fibroblast Transformation

The objective of medium transfer experiments illustrated in Figure 5A was to verify whether the factor that can induce the expression of α-SMA and fibronectin in fibroblasts was released into the medium, acting in an autocrine/paracrine manner. The donor fibroblasts were treated with osteocalcin with or without captopril, presuming Ang II, if any, would be released into the medium with the latter but not the former treatment. These media were then transferred to recipient cells pretreated with captopril, such that even there were osteocalcin in the conditioned media, the cells cannot produce their own Ang II. If osteocalcin triggers Ang II production and release in the donor cells, the addition of the conditioned media would be expected to mimic exogenous Ang II in inducing the expression of remodeling markers.

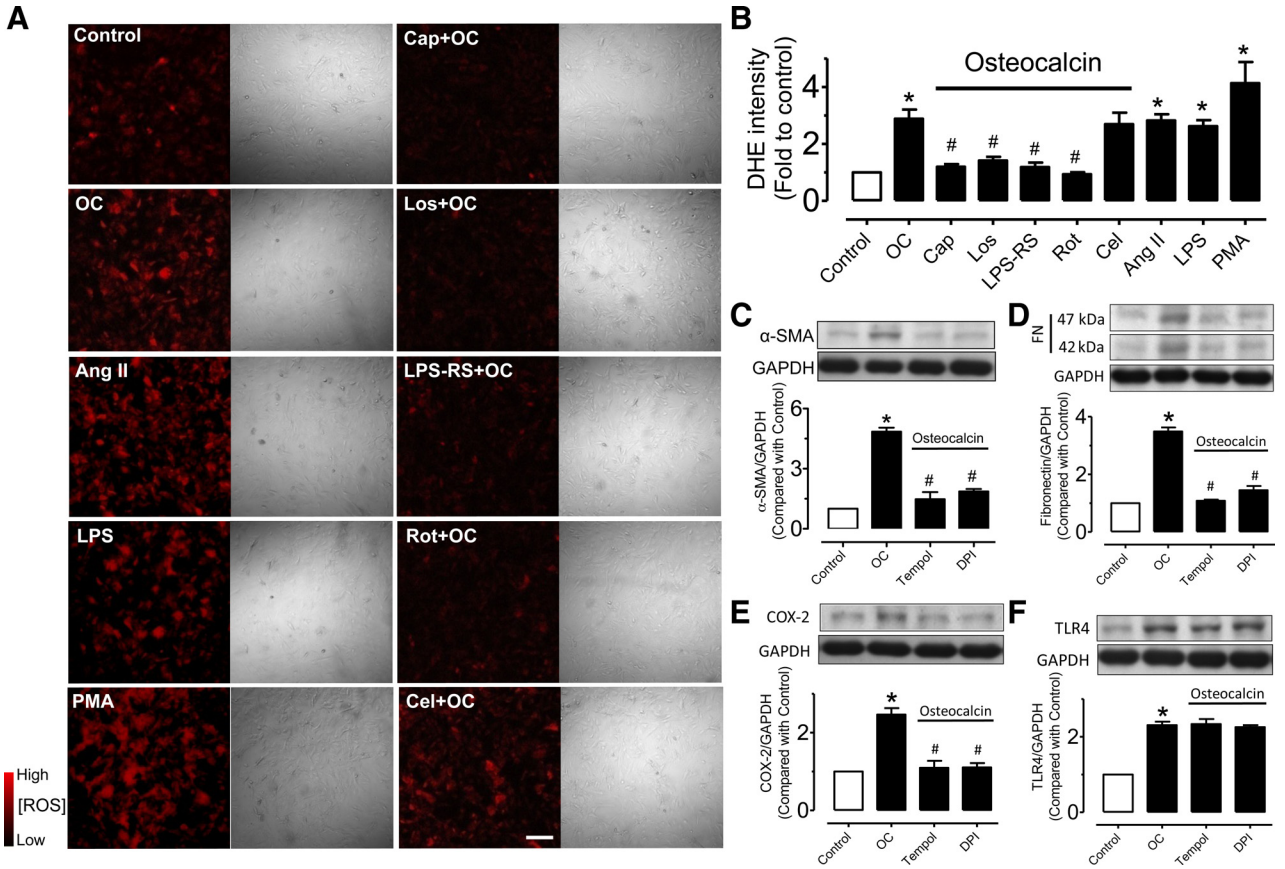

Figure 4. Osteocalcin induces ROS production, which mediates fibroblast transformation. A, Representative images, and **B**, summarized data of DHE fluorescence microscopy on ROS production in the fibroblasts and the effects of various inhibitors (n=5–6). Scale bar denotes 100 μm. **C** through **F**, Effects of ROS scavenger tempol (100 μmol/L) and NADPH oxidase inhibitor DPI (100 nmol/L) on osteocalcin-induced expression of **C**, α-SMA; **D**, FN; **E**, COX-2; and **F**, TLR4 (n=5). One-way ANOVA; *P<0.05 versus control; #P<0.05 versus OC.

The recipient fibroblasts bathed in the conditioned medium from osteocalcin-treated donor fibroblasts exhibited a significant rise in the expression of α-SMA and fibronectin (Figure 5B and 5C). By contrast, conditioned medium from donor fibroblasts pretreated with the ACE inhibitor captopril before osteocalcin exposure did not show the remodeling markers in the recipient cells (Figure 5B and 5C), implying that the released factor is most likely to be Ang II as the increased expression of both markers was paralleled by a markedly elevated amount of Ang II present in conditioned medium

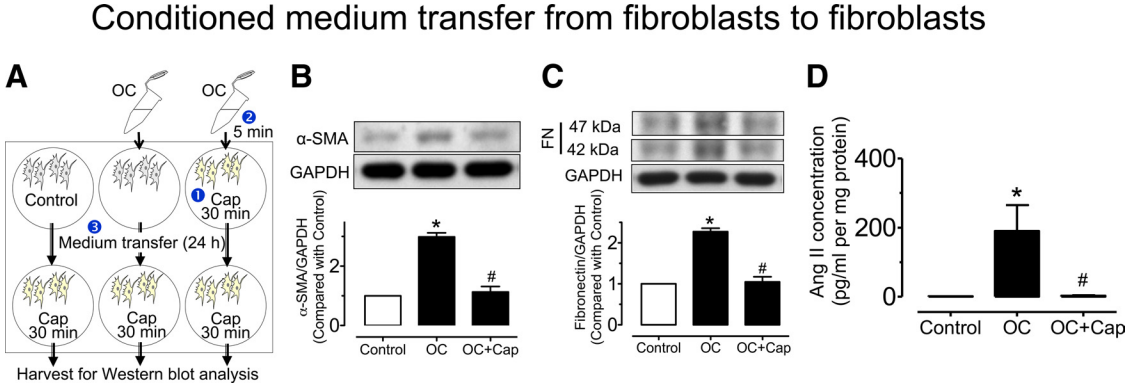

Figure 5. Factor(s) from osteocalcin-treated fibroblasts is transferable and likely to be Ang II. A, Experimental scheme of medium transfer between fibroblasts. The donor fibroblasts were treated with osteocalcin with or without captopril pretreatment, presuming Ang II, if any, would be released into the medium with the latter but not the former treatment. These media were then transferred to recipient cells pretreated with captopril, such that even there were osteocalcin in the conditioned media, the cells cannot produce their own Ang II. The recipient cells are then harvested for Western blot analysis for the expression of α-SMA and FN. **B** and **C**, Western blots showing the effect of conditioned media from osteocalcin-treated donor fibroblasts to captopril-treated recipient fibroblasts on the expression of **B**, α-SMA, and **C**, FN (n=5). **D**, Immunoassay on Ang II levels in the conditioned media from osteocalcin-treated donor fibroblasts (n=5). One-way ANOVA; *P<0.05 versus control; #P<0.05 versus OC.

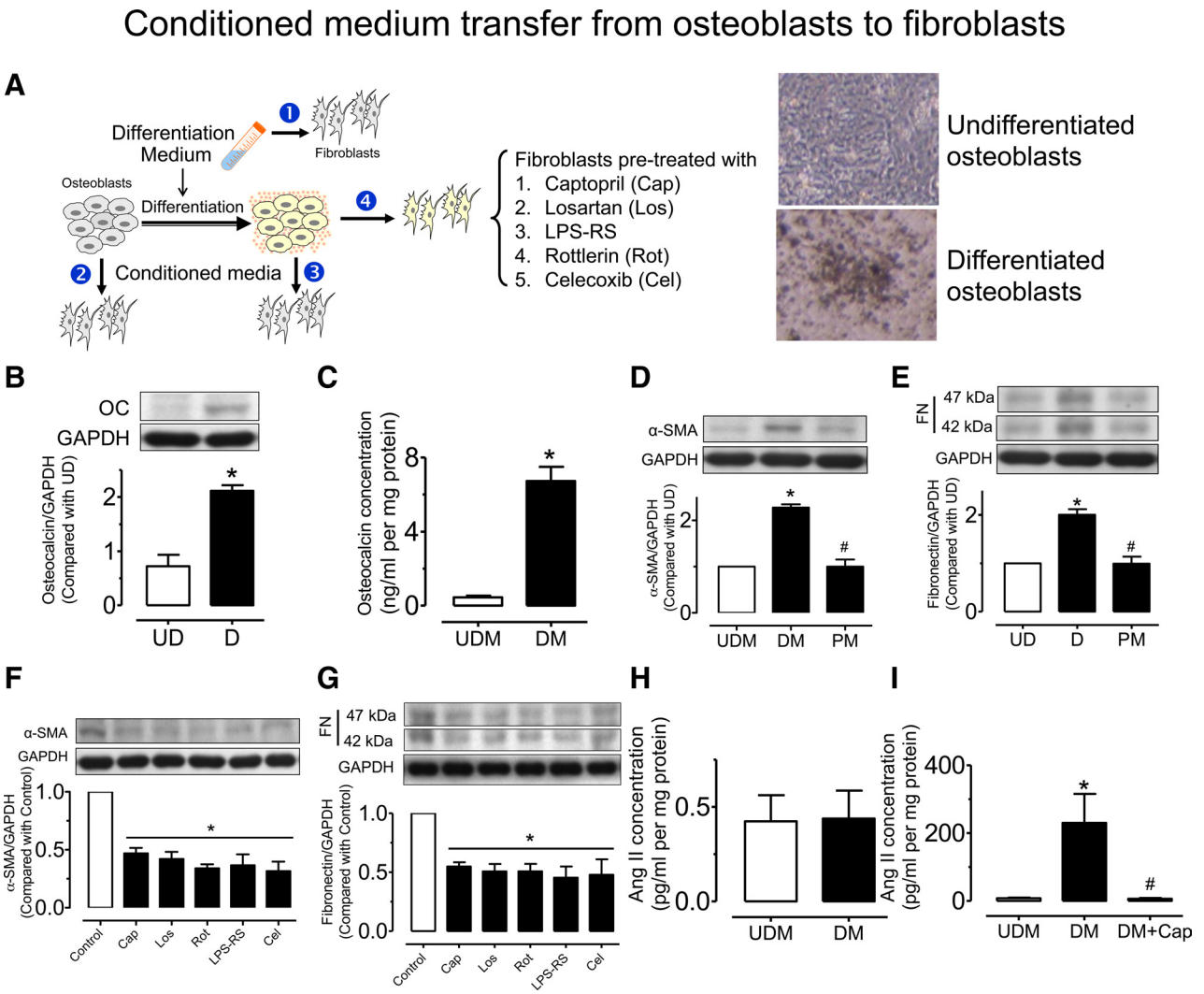

Figure 6. Factor(s) from differentiated osteoblasts transforms fibroblasts. A (left panel), Experimental scheme of medium transfer from osteoblasts to fibroblasts. **A (right)**, Photomicrographs of undifferentiated and differentiated osteoblasts. **B**, Western blot, and **C**, enzyme immunoassay on the osteocalcin levels in osteoblasts and their conditioned media (n=5–6). Student *t* test; **P*<0.05 versus UD or UDM. **D** through **G**, Effect of conditioned media from osteoblasts and various inhibitors on the expression of **D** and **F**, α-SMA, and **E** and **G**, FN in the recipient fibroblasts (n=5). One-way ANOVA; **P*<0.05 versus UDM or control (conditioned medium from differentiated osteoblasts); #*P*<0.05 versus DM. Enzyme immunoassay on the Ang II levels in the conditioned media from osteoblasts **H**, before, and **I**, after transferring to recipient fibroblasts (n=5). One-way ANOVA; **P*<0.05 versus UDM; #*P*<0.05 versus DM. UDM indicates medium from undifferentiated osteoblasts; DM, medium from differentiated osteoblasts; and PM, native differentiation medium without incubation with osteoblasts.

from osteocalcin-treated donor fibroblasts and the Ang II rise was eliminated by captopril (Figure 5D).

The second series of experiments was designed to give further support to the role of osteocalcin in triggering the upregulated expression of α-SMA and fibronectin in fibroblasts using conditioned medium collected from osteoblasts, which represent the only cell type that normally releases osteocalcin.[30] Conditioned media were collected from both undifferentiated and differentiated rat osteoblasts, UMR-106, and then transferred to the fibroblasts (Figure 6A, left). Differentiated osteoblasts, appearing clumped and mineralized (Figure 6A, right), expressed and released osteocalcin, as evidenced by the elevated osteocalcin levels in the cells and conditioned medium (Figure 6B and 6C). This conditioned medium upregulated the expression of α-SMA and fibronectin, whereas native differentiation medium and the conditioned medium from undifferentiated osteoblasts had no effect on the fibroblasts (Figure 6D and 6E). We then proceeded to identify the mediators of the conditioned medium-triggered fibroblast transformation using captopril, losartan, LPS-RS, rottlerin, and celecoxib; all of which were preincubated for 30 minutes with the recipient fibroblasts before the addition of the conditioned medium from the differentiated osteoblasts (Figure 6A, left panel). Each of these inhibitors reversed the conditioned medium-stimulated expression of α-SMA and fibronectin (Figure 6F and 6G). While the conditioned media from either undifferentiated or differentiated osteoblasts before transferring to the fibroblasts contained an extremely low level of Ang II (<0.5 pg/mL per mg protein, Figure 6H), the medium from differentiated but not undifferentiated osteoblasts harvested after transfer and a 24-hour incubation in the recipient fibroblasts exhibited an

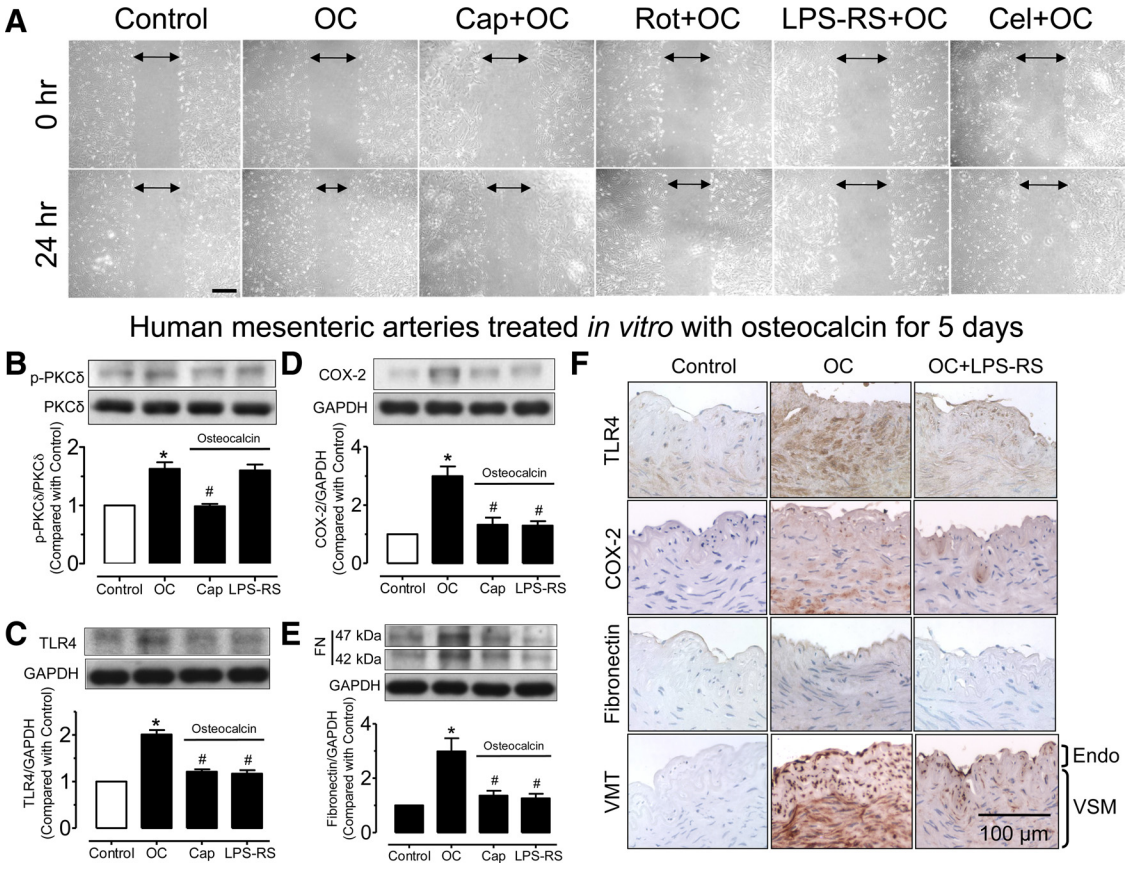

Figure 7. Osteocalcin induces fibroblast migration and expression of remodeling markers in human mesenteric arteries. A, Wound-healing assay showing rat fibroblast migration in response to osteocalcin and the effects of various inhibitors (n=5). Scale bar denotes 200 μm. Osteocalcin-induced (**B**) PKCδ phosphorylation and expression of **C**, TLR4; **D**, COX-2; and **E**, FN and the effects of ACE inhibitor captopril (Cap, 100 nmol/L) and TLR4 antagonist (LPS-RS, 10 ng/mL) in human mesenteric arteries (n=3). One-way ANOVA; *$P<0.05$ versus control; #$P<0.05$ versus OC. **F**, Immunohistochemistry on TLR4, COX-2, FN, and VMT in human mesenteric arteries exposed to osteocalcin (10 nmol/L) for 5 days (n=5–7).

≈200-fold increase in captopril-sensitive Ang II level (Figure 6I), demonstrating that the skeletal hormone osteocalcin secreted from osteoblasts is capable of stimulating the production and release of Ang II in the fibroblasts and the latter in turn triggers fibroblast transformation.

Osteocalcin Induces Fibroblast Migration

Fibroblasts are equipped with slight contractile and migratory capability after expressing α-SMA. We performed a wound-healing assay to demonstrate the functional relevance of fibroblast transformation. Twenty-four-hour treatment with osteocalcin concentration-dependently stimulated fibroblasts to migrate (Online Figure VIA). The osteocalcin-induced (10 nmol/L) migration was reversed by captopril, LPS-RS, rottlerin, and celecoxib (Figure 7A and Online Figure VIB).

Osteocalcin Stimulates the Expression of Remodeling Markers in Human Arteries

Finally, to elucidate the relevance of the osteocalcin-triggered remodeling pathway in human arteries, we conducted tissue culture on the mesenteric arteries from normotensive and nondiabetic patients. Five-day in vitro treatment with osteocalcin (10 nmol/L) augmented the phosphorylation of PKCδ and expression levels of TLR4, COX-2, and fibronectin, all of which were inhibited by captopril, whereas LPS-RS only reversed the expression of TLR4, COX-2, and fibronectin but not PKCδ phosphorylation (Figure 7B through 7E). This again supports that PKCδ modulates TLR4 but not vice versa. Immunohistochemistry also showed increased staining for TLR4, COX-2, and fibronectin across the vascular wall in osteocalcin-treated human arteries, and their expression was reduced by coincubation of LPS-RS (Figure 7F). The heavy staining of vimentin suggests the presence of fibroblasts in the inflamed vascular wall (Figure 7F).

Discussion

Osteocalcin has been implicated in the pathogenesis of vascular remodeling; however, the mechanisms of its action remain elusive. The detection of osteocalcin expression in the neointima of renal arteries from diabetic patients directed us to hypothesize that osteocalcin may contribute to fibroblast transformation, a major feature in the modern concept of vascular remodeling according to the "outside-in" theory. The key findings of the present investigation are as follows: (1) osteocalcin is associated with arterial neointimal growth; (2) proinflammatory markers such as TLR4 and COX-2 are coexpressed with the remodeling proteins α-SMA and fibronectin in the human neointimal lesions; (3) osteocalcin promotes fibroblasts transformation to myofibroblasts as

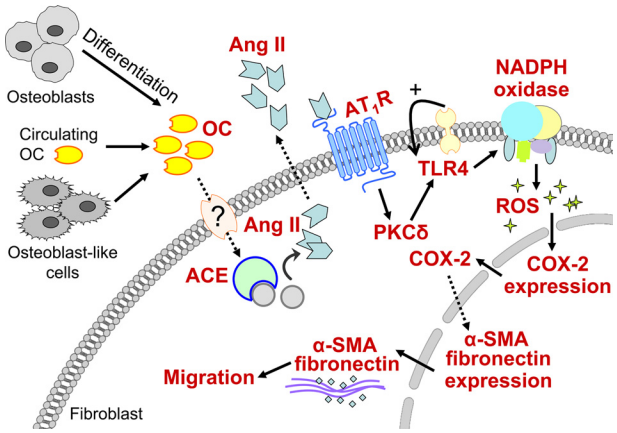

Figure 8. Schematic diagram proposing the mechanism of osteocalcin-induced fibroblast transformation. Osteocalcin induces Ang II production, which acts as an autacoid in the fibroblasts to activate PKCδ/TLR4/ROS/COX-2 signaling cascade to mediate fibroblast transformation. TLR4 stimulation further upregulates TLR4 expression. OC indicates osteocalcin; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT_1R, angiotensin II type 1 receptor; PKCδ, protein kinase Cδ; TLR4, toll-like receptor-4; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; and α-SMA, α-smooth muscle actin.

evidenced by the increased expression of α-SMA and fibronectin, which enables the transformed fibroblasts to migrate and contract; and (4) osteocalcin stimulates the production and release of Ang II in fibroblasts, which acts in an autocrine manner as an initial trigger to activate PKCδ/TLR4/ROS/COX-2 signaling cascade, thus mediating fibroblast transformation. This study highlights the interaction and cross-talk between the skeleton hormone osteocalcin and the vascular wall in the pathogenesis of vascular dysfunction (Figure 8 and Online Figure VII).

The pathophysiology of vascular neointima formation is intricate and multifaceted. It has long been believed that medial smooth muscle cells (SMCs) play an integral role in the initiation of intimal lesions through their migration toward and proliferation in the intimal layer, thus increasing the lesion volume.[31] This is essentially inferred from clear evidence of increased expression of SMC markers α-SMA and SM22α in the neointima.[32] Nonetheless, it is also probable that the neointima harbors a heterogeneous cell population. Previous studies suggest that after phenotypic transformation to myofibroblasts, adventitial fibroblasts acquire contractile capability and contribute to intimal thickening.[4,5] Such response is attributed to the change in the cellular components of the adventitial fibroblasts, characterized by augmented matrix synthesis and induced expression of cytoskeleton proteins such as α-SMA.[2] It is worth noting that the development of neointimal hyperplasia is concomitant with a substantial increase in the thickness of the adventitia.[1] Indeed, a number of studies suggest that the adventitia may become an active component on balloon-induced injury or angioplasty, and its activation is implicated in vascular remodeling and restenosis.[1,33] The adventitia represents the major site where proliferating cells are situated in porcine coronary arteries after angioplasty.[6] The number of proliferating cells decreases in the adventitia 1 week after angioplasty; instead, a majority of them are found in the neointima.[6] Likewise, studies on animal models of hypercholesterolemia and hypertension also show that adventitial remodeling precedes intimal and medial remodeling.[34] Since adventitial fibroblasts are the primary cell type present in the adventitia, it is likely that they take a pivotal lead in the initiation of neointima formation on injury. Li et al[35] provided direct in vivo evidence by showing the migration of adventitial fibroblasts to the neointima in the rat endoluminal vascular injury model with the ß-galactosidase (ß-LacZ) reporter gene. At 7 and 14 days after injury, the ß-LacZ–positive cells detected in the neointima were also found to be α-SMA positive, indicating that the migratory adventitial fibroblasts had been differentiated to myofibroblasts. Collectively, these results reveal that adventitial fibroblasts possess a migratory ability to translocate to the intimal layer, thereby contributing to neointimal lesion growth. Based on this premise, the present study examines how osteocalcin participates in the transformation of fibroblasts to myofibroblasts, which is an important step preceding neointimal hyperplasia.

One of the major novel findings of this study is that adventitial fibroblasts are able to produce and secrete Ang II on osteocalcin stimulation, breaking the traditional concept of the relatively quiescent phenotype of adventitial fibroblasts. Ang II is associated with the narrowing of arterial lumen as a result of neointima formation.[36] Consistently, the RAS is activated in vascular neointima in response to injury, manifested by the increased ACE activity and the upregulated expression of AT_1R.[37] This may explain why ACE inhibitors can prevent injury-induced neointima formation and restenosis.[38] Experimental results support the findings of clinical trials by showing that RAS blockade inhibits vascular remodeling after injury.[39,40] Although Ang II is known to promote myofibroblast migration,[41] our study using the conditioned medium from differentiated osteoblasts further points to that osteocalcin acts as a natural stimulant to release Ang II in the adventitial fibroblasts and Ang II subsequently functions as an autacoid to trigger fibroblast differentiation to myofibroblasts via the induction of α-SMA expression and upregulation of fibronectin. Such effect is compromised by the treatment with ACE inhibitor and AT_1R blockers, suggesting that osteocalcin stimulates the RAS to upregulate the remodeling markers. The vaso-protection of RAS blockade observed in clinical studies may partially be attributed to their suppression of fibroblast transformation and thus the retardation of neointima formation. The present results thus reveal the additional benefits of ACE inhibitors and AT_1R blockers in preventing neointima formation besides blood pressure–lowering effect. Although we cannot completely rule out if any minor contributions of other cytokines released by the differentiated osteoblasts, we attempt to propose that osteocalcin-induced production of Ang II is the main trigger for adventitial fibroblast transformation based on the following 3 observations. First, osteoblasts are the main cell type able to release osteocalcin and we confirm an increase in the osteocalcin level in the conditioned medium from differentiated osteoblasts. Second, conditioned medium from differentiated osteoblasts stimulated expression of α-SMA and fibronectin, which are sensitive to captopril and losartan,

indicating the prime involvement of the RAS. Third, the native medium from differentiated osteoblasts contains a low level of Ang II, which is elevated profoundly after being transferred to fibroblasts and the Ang II production is inhibited by captopril before exposure, thus confirming that fibroblasts are the source of Ang II. These all indicate the critical relationship between Ang II production from fibroblasts under the natural stimulant of osteocalcin derived from osteoblasts. Since the enzyme responsible for osteocalcin production is unknown, this renders ultimate confirmation of the contribution of osteocalcin by siRNA knock-down of the enzyme unfeasible. However, together with the similar results using exogenous osteocalcin, the conditioned medium-stimulated expression of remodeling markers is very likely attributed to osteocalcin.

TLR4 is implicated in vascular remodeling. Upregulated TLR4 expression is found in human and mouse vein graft remodeling.[42] A remarkable reduction in vein graft hyperplasia and outward remodeling after vein grafting in TLR4 knock-out mice further corroborates a contributory role of TLR4 in vascular dysfunction. In fact, TLR4 activation is associated with vascular injury and inhibition of TLR4 signaling pathway reduces neointima formation in response to injury in mouse carotid artery.[43] Earlier studies show that adventitial fibroblasts express functional TLR4 in human atherosclerotic arteries[12] and TLR4 is involved in cuff-induced neointima formation, leading to outward arterial remodeling as a result of augmented cell migration and matrix turnover.[44] Consistently, our results show a positive immunohistochemical staining of TLR4 in the neointima of human renal arteries. It has been reported that adventitial LPS application on rat femoral arteries contributes to intimal lesions.[45] Taken together, earlier studies suggest a possible linkage between TLR4 activation and adventitial fibroblast migration. Our findings concur with the hypothesis that osteocalcin stimulates the expression of α-SMA and fibronectin, which is sensitive to both TLR4 neutralizing antibody and specific antagonist. This is probably the first report that delineates the role of TLR4 and its signaling cascade in the osteocalcin-induced myofibroblast formation.

The present findings reveal an important contribution of ROS as a downstream mediator of TLR4 in fibroblast transformation. Not limiting to intimal surface, ROS can also be generated in the adventitia on inflammation or injury.[1] ROS overproduction is indicative of local inflammation, which has been associated with elevated fibroblast proliferation and migration,[41] augmented matrix production,[46] and neointima formation.[47] In fact, TLR4 activation is closely related to inflammation.[12] It is likely that the TLR4 activation in adventitial fibroblasts attracts monocytes through the production of inflammatory cytokines, which promotes fibroblast migration and proliferation (reviewed by Siow and Churchman).[33] Examination of osteocalcin-stimulated TLR4 activation provides critical insights into its role in inflammation and the pathogenesis of vascular neointima formation. The present results indicate that TLR4 activation may affect NADPH oxidase-dependent ROS overproduction. It is conceivable that TLR4 antagonists and antioxidative drugs might be effective therapeutic options to ameliorate intimal remodeling.

Neointima formation, subsequently accompanied by the recruitment of monocytes and accumulation of macrophages and lipids, may contribute to the pathogenesis of atherosclerosis. Existing evidence indicates an interplay among bone-associated proteins such as osteocalcin, neointima formation, and atherosclerosis,[14,15] which may be a consequence of active osteogenesis in vascular tissues. A recent study points out that osteocalcin stimulates cartilage and vascular calcification in rats through the hypoxia-inducible factor 1α-dependent mechanism on glycolytic breakdown of glucose.[48] The sources of such osteoblast-like cells remain unclear; therefore, the identification of osteocalcin in human intimal lesion has several important implications about its origin. First, active osteogenesis by osteoblast-like cells is demonstrated in the early stage of inflamed atherosclerotic aortae.[15] In this regard, some studies indicate that osteoblastic cells are formed as the vascular SMCs (VSMCs) undergo differentiation to acquire osteogenic phenotype in response to oxidative stress or bone morphogenic proteins.[49] Such osteoblastic differentiation of VSMCs may account for the presence of osteoblast-like cells in atherosclerotic tissues. Interestingly, immunohistochemical staining for Ang II in our study showed that VSMCs are major producers of Ang II in human mesenteric arteries exposed to osteocalcin, suggesting VSMCs as a source of Ang II to trigger fibroblast transformation in addition to the autocrine Ang II release from the stimulated fibroblasts. Whether or not the VSMCs are, on the other hand, transformed to osteoblast-like cells deserves further exploration. Second, it has been suggested that progenitor cells are responsible for the onset of atherosclerosis.[50] In fact, progenitor cells that are identified in the adventitia of human pulmonary arteries are capable of differentiating to SMCs and osteogenic cells in vitro.[51] However, whether the adventitial progenitors that are transformed to osteoblasts are capable of producing osteocalcin after injury or during inflammatory response remains unsolved. It is therefore of interest to examine the bone-vascular axis to unravel if the upregulated osteocalcin expression in neointima or atherosclerotic plaque is due to bone cells, osteoblast-like cells, or progenitors in vasculature.

Although the osteogenic process is associated with vascular dysfunction, there is a paucity of studies elucidating its precise mechanisms. The effect of osteocalcin in neointimal growth and vascular remodeling remains unexplored. The novel findings in the present study indicate a positive contribution of osteocalcin in neointima formation through an adventitial activation of Ang II/PKCδ/TLR4/ROS/COX-2 signaling pathway, albeit the actual osteocalcin receptor is yet to be identified. The present results serve as an important conceptual framework for further exploration of the skeleton-vascular interaction that is associated with the exaggeration of vascular complications. The concept of osteogenic involvement in vascular remodeling offers novel strategies to retard neointima formation and atherosclerosis. Elucidation of the key mediators in this cascade may represent a new target for therapies in the alleviation of vascular remodeling.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Vascular inflammation can originate from the adventitia and progress toward the intima.
- Adventitial fibroblasts can differentiate to myofibroblasts in response to vascular injury.
- Human atherosclerotic plaque formation resembles osteogenesis.
- Osteocalcin and toll-like receptor 4 have been individually implicated in arterial remodeling.

What New Information Does This Article Contribute?

- Osteocalcin stimulates the production and the release of angiotensin II in fibroblasts.
- Osteocalcin and angiotensin II transform adventitial fibroblasts to myofibroblasts depending on toll-like receptor 4 and cyclooxygenase-2.
- Osteocalcin-treated fibroblasts gain the capacity to contract and migrate.

In contrast to the current view that vascular dysfunction progresses from "inside-out," recent evidence suggests an "outside-in" hypothesis, proposing that inflammation can originate from the adventitia and develop toward the intima, resulting in vascular wall thickening. Vascular remodeling events such as neointima formation are characterized by the acquisition of migratory and proliferative ability of fibroblasts after their transformation into myofibroblasts. Toll-like receptor 4 expression has been reported in human and murine arterial lesions and intimal hyperplasia. Although cumulating evidence implies a cross-talk between bone pathology and cardiovascular diseases, there is a lack of clear mechanism on how bone-associated proteins such as osteocalcin affect the vasculature. The present study shows that in human neointimal lesions, the proinflammatory markers toll-like receptor 4 and cyclooxygenase-2 are coexpressed along with the proteins involved in remodeling such as α-smooth muscle actin and fibronectin. The study also led to the identification of a mechanistic pathway in which osteocalcin triggers adventitial fibroblast to produce and release angiotensin II. This in turn activates protein kinase Cδ, toll-like receptor 4, and cyclooxygenase-2 to transform fibroblasts into myofibroblasts. These findings support the pathogenic linkage between the skeletal hormone and vascular remodeling.

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Supplemental Material

DETAILED METHODS

Animals

This investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). This study was approved by the Experimental Animal Ethics Committee, the Chinese University of Hong Kong. Male Sprague-Dawley rats weighing 260-280 g were supplied from the Laboratory Animal Services Center, the Chinese University of Hong Kong.

Processing of human renal and mesenteric arteries

To study the relevance of remodeling markers under a pathological condition, renal arteries were obtained from patients without or with diabetes (fasting plasma glucose level ≥ 7.0 mmol/L) but normal blood pressure (systolic and diastolic blood pressure <140 mmHg and <90 mmHg respectively). Mesenteric arteries were all from normotensive and non-diabetic patients for *in vitro* tissue culture experiments. Renal arteries were harvested from patients undergoing nephrectomy due to renal malignancy, kidney infection or non-functioning and were preserved in paraffin for immunohistochemistry. Mesenteric arteries were collected from the resected mesenteric tissues from patients operating for colon cancer. Only arteries that are reasonably distant from the tumor were used in the study to prevent a high basal expression level of inflammatory markers. The arteries were cultured with osteocalcin (10 μmol/L) for 5 days in DMEM supplemented with 10 % FBS. Inhibitors, when used, were added 30 min before exposure to osteocalcin. The arteries were then frozen for Western blot analysis or fixed for immunohistochemistry.

Primary culture of rat aortic adventitial fibroblasts and adventitia

Thoracic aortae isolated from Sprague-Dawley rats were dissected under sterile conditions. After removal of the perivascular adipose tissue, the aortae were cut open and stripped of the intima and media. The adventitia was subjected to digestion by 0.2 % Type 1A collagenase (Sigma, St. Louis, MO) in PBS at 37^{o}C for 15 min. Thereafter, the suspension was centrifuged at 1500 rpm for 10 min. The isolated cells were re-suspended in Dulbecco's Modified Eagle's Media (DMEM, Gibco, Grand Island, NY) supplemented with 10 % fetal bovine serum (FBS) (Gibco, Grand Island, NY), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY). Cells were allowed to settle and the medium was changed 24 hours afterwards. Only adventitial fibroblasts from the first 2-3 passages were used for the study. Cells were serum starved for 48 hours prior to drug treatment. Identity of the primary culture of rat aortic adventitial fibroblasts was confirmed by a positive staining to a fibroblast marker, vimentin, and negative staining to PECAM-1 and α-SMA, the markers for endothelial cells and smooth muscle cells, respectively. The same medium was used to culture adventitia mechanically tore from the rat aortae.

Flow cytometry on the primary culture of cells

The identity of cells was confirmed with flow cytometry (FACS analysis). One million cells per sample were adopted for FACS analysis for each marker. For surface markers of CD33 and PECAM-1, cells were blocked with 10% normal goat serum to prevent non-specific binding. The cells were allowed to incubate on ice for 30 min with occasional shaking. After washing in PBS with 1% BSA, the cells were resuspended in 250 μL PBS with 1% BSA. Fifty μL of the cell suspension were mixed with 50 μL (0.5 μg) primary antibody and incubated on ice for 30 min with occasional shaking. After washing, 0.5 μg appropriate secondary antibody was added and the cells were incubated on ice for 30 min with occasional shaking. Cells were then washed and fixed in 1% paraformaldehyde and FACS was performed on BD LSRFortessa cell analyzer (Franklin Lakes, New Jersey, USA). For intracellular markers of vimentin, P4H and α-SMA, a permeabilization step of incubating the cells in 1.5% Tween-20 for 15 min at room temperature was performed prior to blocking with normal goat serum.

Culture and differentiation of osteoblasts

Rat osteoblasts, UMR-106 (American Type Culture Collection), were a kind gift from Dr Ling Qin, the Chinese University of Hong Kong. The cells were grown and proliferated in DMEM supplemented with 10 % FBS. Upon 90 % confluence, the cells were allowed to differentiate to mature osteoblasts with 50 μg/mL L-ascorbic acid and 10 mmol/L β-glycerophosphate. Conditioned medium was collected after 4-day differentiation for transfer experiments to the fibroblasts and the osteoblasts

were harvested for determination of osteocalcin production by Western blotting.

Transfer experiments of conditioned medium
Conditioned medium transfer was conducted from fibroblasts to fibroblasts and from osteoblasts to fibroblasts. In the first series of experiments between fibroblasts, donor fibroblasts were exposed to osteocalcin (10 nmol/L) for 5 min, and conditioned medium was either collected for determination of Ang II level or transferred to the recipient fibroblasts pre-treated with captopril (100 nmol/L) for 24-hour incubation (Figure 5A). In the second series of experiments between osteoblasts and fibroblasts, native differentiation medium, conditioned medium from undifferentiated or differentiated osteoblasts was transferred to the fibroblasts (Figure 6A). The medium was collected for Ang II and osteocalcin immunoassay, and osteoblasts were examined for the production of osteocalcin.

SiRNA silencing of PKCδ and TLR4 in adventitial fibroblasts
Adventitial fibroblasts were transfected with siRNA targeting PKCδ (siPKCδ) or TLR4 (siTLR4) by electroporation using an Amaxa basic nucleofector kit (Lonza, Germany).[1] Briefly, fibroblasts grown to confluence were trypsinized, and resuspended in 100 µL basic nucleofector solution. The cell suspension was transferred to an electroporation cuvette containing either 60 pmol scramble siRNA, 30 pmol siPKCδ (Ambion) or 60 pmol siTLR4 (Thermo Scientific). The cells were electroporated with the Amaxa Nucleofactor apparatus and then plated in 6-well plates containing pre-warmed complete DMEM. Medium was changed after 24 hours, and then serum-deprived for 24 hours before exposing to osteocalcin (10 nmol/L) for another 24 hours.

Western blot analysis
Western blotting was performed as described.[2] Tissues or cells were homogenized in an ice-cold RIPA lysis buffer containing a cocktail of protease inhibitors. Cell lysates were centrifuged at 20,000 g for 20 min at 4 ^{o}C. Protein concentration of the supernatants was determined using the Lowry method (Bio-rad, Hercules, CA, USA). Equal amount of protein samples were electrophoresed on a 10 % SDS-polyacrylamide gel and then transferred onto an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked by 5% non-fat milk in 0.05% Tween-20 phosphate-buffered saline (PBST), then incubated overnight at 4°C with primary antibodies including α-SMA, fibronectin, AT_1R (Abcam, Cambridge, UK), COX-2, TLR4, ACE, osteocalcin and PECAM-1 (Santa Cruz Biotechnology, CA, USA), COX-1 (Cayman, Ann Arbor, MI, USA), PKCδ and phosphorylated PKCδ (Cell signaling, Beverly, MA, USA), P4H (Millipore-Chemicon, Billerica, MA, USA) and renin (SWANT, Switzerland) followed by a HRP-conjugated swine anti-rabbit or anti-mouse IgG (DakoCytomation, Carpinteria, CA, USA). The membrane were developed with an enhanced chemiluminescence detection system (ECL reagents, Amersham Pharmacia), and finally exposed to X-ray films. Equal loading of protein was confirmed with the housekeeping GAPDH protein.

Immunohistochemistry
Human renal arteries and osteocalcin-treated human mesenteric arteries were fixed for immunohistochemistry for osteocalcin, α-SMA, vimentin, TLR4, COX-2 and/or Ang II. The arteries were fixed in 4 % paraformaldehyde, embedded in wax and cut into in 5-µm sections, which were treated with 1.4 % hydrogen peroxide for 30 min. The sections were boiled in 0.1 M citric buffer for 45-60 s, blocked with 5 % normal donkey serum and incubated with primary antibodies diluted in 2 % BSA in PBS at 4 ^{o}C overnight. After washing PBS, the sections were exposed to appropriate biotinylated secondary antibodies for 1 hour at room temperature and then peroxidase-conjugated streptavidin for another 1 hour. The sections were developed with DAB (Vector Laboratories, California, USA) according to the manufacturer's instruction and nuclei were counter-stained with hematocxylin. The images were captutred with Leica DMRBE microscope with SPOT Advanced software (Version 3.5.5).

Immunofluorescence microscopy
Co-existence of prolyl 4 hydroxylase (P4H) and α-SMA and the formation of myofibers were examined by immunofluorescence confocal microscopy. Briefly, the cells were fixed with 4% paraformaldehyde after drug treatment. Primary antibodies against P4H and α-SMA were incubated overnight at 4^{o}C. After several washes in PBS, the cells were then incubated with Alexa Fluor 488 and 546 IgG (Invitrogen) for 1 hour at room temperature. After cover-slipping, the cells were observed under the fluorescence microscope Nikon Live Cell Imaging System (Eclipse Ti-E) using a 60x oil lens. Images were acquired with the MetaMorph software.

Enzyme immunoassay for Ang II and osteocalcin
Conditioned media bathing human mesenteric arteries, fibroblasts and osteoblasts were harvested for the immunoassay for Ang II (SPIbio, Montigny Le

Bretonneux, France) and/or osteocalcin (Biomedical Technologies Inc., Stoughton, MA) according to the manufacturers' instruction. Extraction was performed on the media with phenyl-cartridges prior to Ang II assay.

DHE fluorescence microscopy and electron paramagnetic resonance (EPR) on ROS production

Detection of ROS generation was performed with DHE (Molecular Probes)[1] and EPR[3,4]. For DHE fluorescence, adventitial fibroblasts after drug treatments were incubated with DHE for 15 min at 37°C. The unbound fluorescence dye was removed by washing the cells several times with normal physiological saline solution (NPSS) containing 140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, and 5 mmol/L HEPES (pH 7.4). Fluorescence was measured under the confocal microscopy system Olympus Fluoview FV1000 (Olympus America Inc, Melville, NY) with excitation wavelength of 515 nm and emission wavelength of 585 nm, with the objective UMPlanFI 10x/0.30. DHE fluorescence intensity was analysed by Olympus Fluoview software (version 1.5; FV10-ASW1.5). Results from 4 separate experiments were presented as the fold change compared with control. As to EPR measurement, cells after treatment were incubated in tyrode solution containing 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (TEMPONE-H, a spin trapping agent, 100 μmol/L, Alexis Biochemical Corp., San Diego, CA, USA) and a transition metal chelator diethylenetriaminepentaacetic acid (DTPA, 100 μmol/L, Sigma-Aldrich) for 20 minutes to trap the ROS produced. The cells were then scrapped and harvested together with the tyrode solution and placed into glass micropipettes for X-band EPR spectra detection at 21°C using an EMX EPR spectrometer (Bruker BioSpin GmbH, Siberstreifen, Rheinstetten/Karlsruhe, Germany) with settings previously described[3,4].

Wound healing assay

Adventitial fibroblasts were seeded in a 12-well culture plate and serum-deprived medium for 48 hours after reaching 90% confluence. A single wound was incised across the cell monolayers with a sterile plastic pipette tip. The cell debris was removed by rinsing with PBS. The wound was observed under a light microscope (Olympus) at 0 hour (before osteocalcin exposure) and 24 hours (after osteocalcin addition) and images were captured. The perpendicular distance between the wound edges at 0 and 24 hours after was measured by the Image J software (National Institute of Health, Bethesda, MD). Migration rate was expressed as migration distance/time (μm/h). Each set of experiments was repeated for 4 times.

Statistical analysis

Data are means ± SEM of n experiments and analyzed with Student's *t* test or one-way ANOVA followed by the Bonferroni post hoc test when more than 2 treatments were compared (Graphpad Prism Software, version 4.0). $P<0.05$ was considered statistically significant.

Chemicals

Osteocalcin and εV1-2 were purchased from AnaSpec (Fremont, CA, USA). Angiotensin II, losartan, ZD 7155, GF109203X, rottlerin, sc-560, Go 6976, DuP-697 and phorbol 12-myristate 13-acetate were purchased from Tocris (Avonmouth, UK). LPS, indomethacin, tempol and DPI were purchased from Sigma-Aldrich (St Louis, Mo, USA). Captopril was from Sigma-RBI (Natick, MA, USA). TLR4 neutralizing antibody was from eBioscience (San Diego, CA, USA). LPS-RS was purchased from InvivoGen (San Diego, CA, USA). Celecoxib was from Pfizer. Osteocalcin, εV1-2, angiotensin II, ZD 7155, LPS, tempol, TLR4 neutralizing antibody and LPS-RS were dissolved in distilled water while others in dimethyl sulphoxide (DMSO, Sigma-Aldrich).

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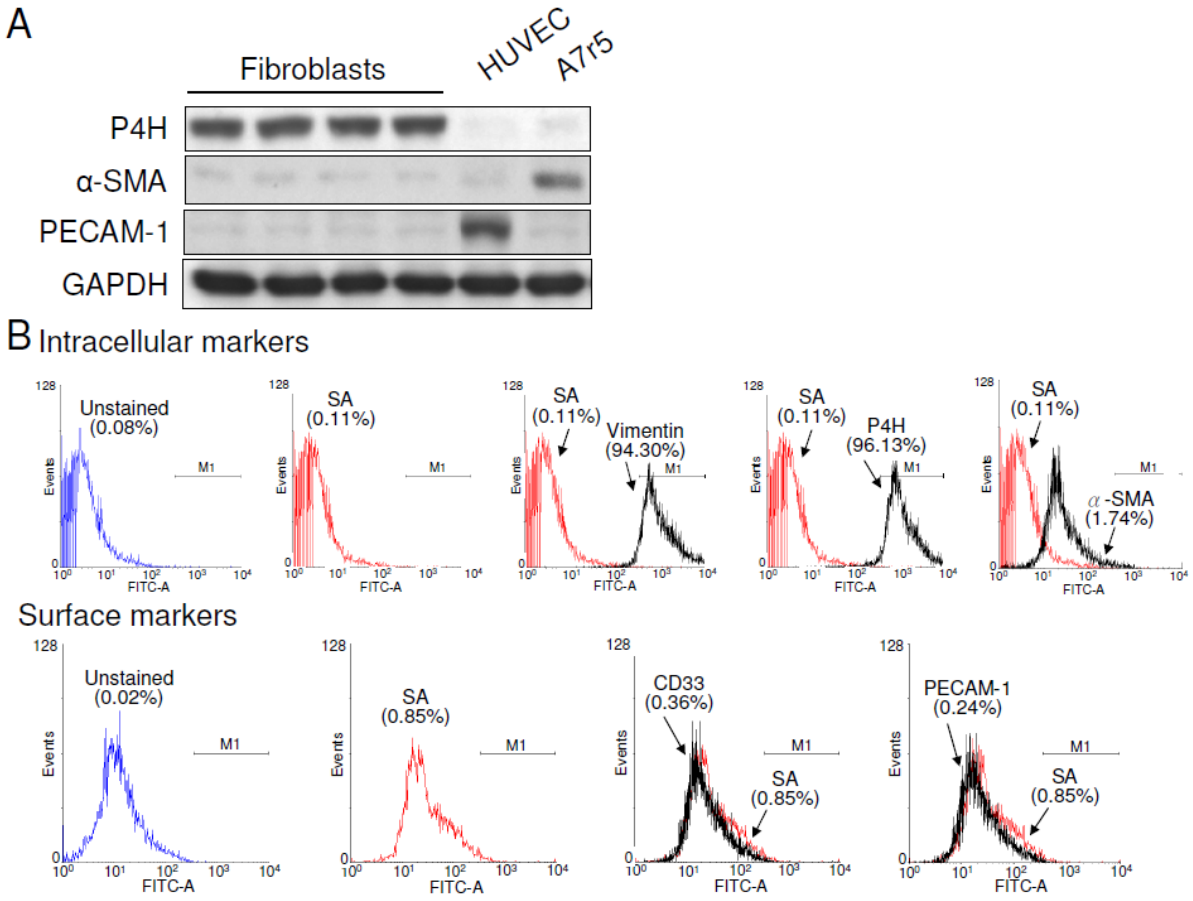
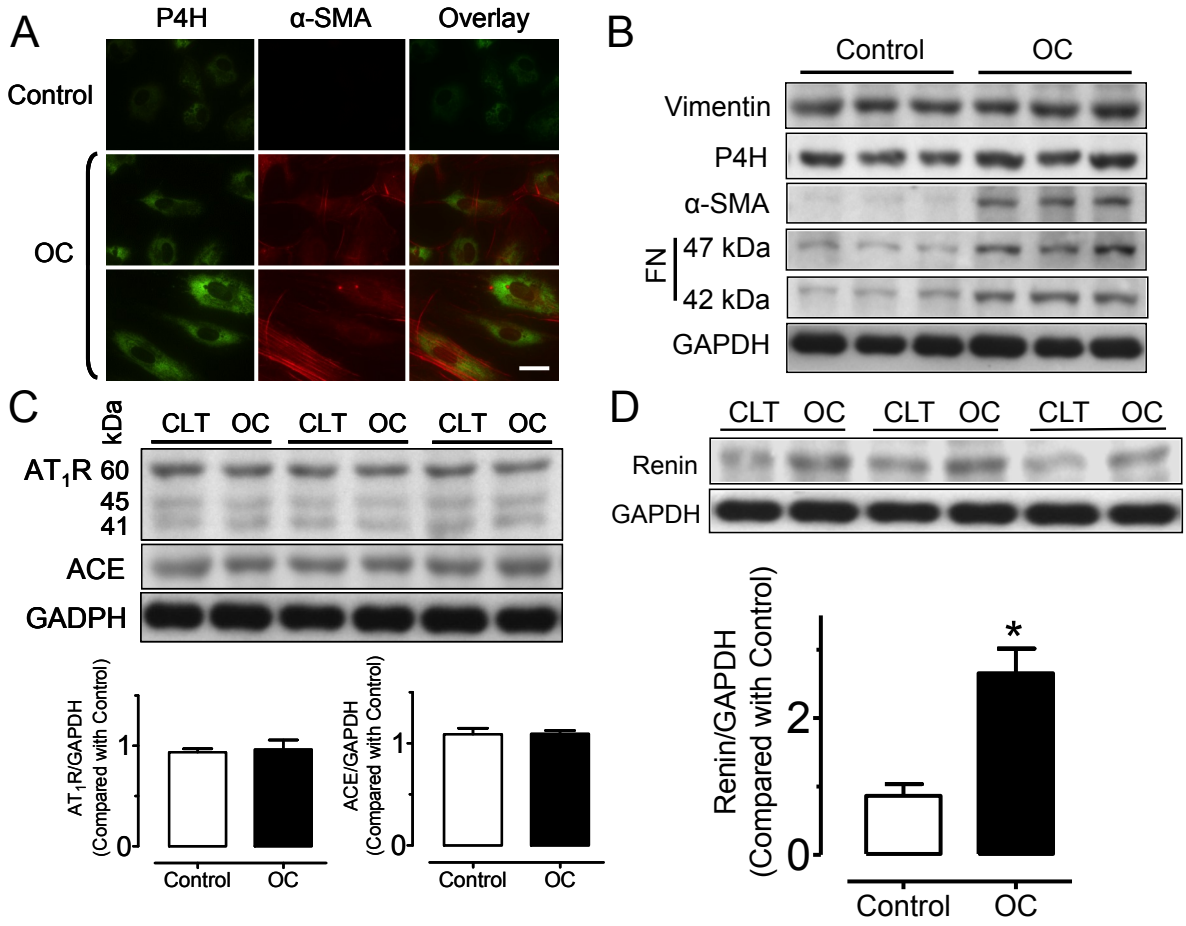
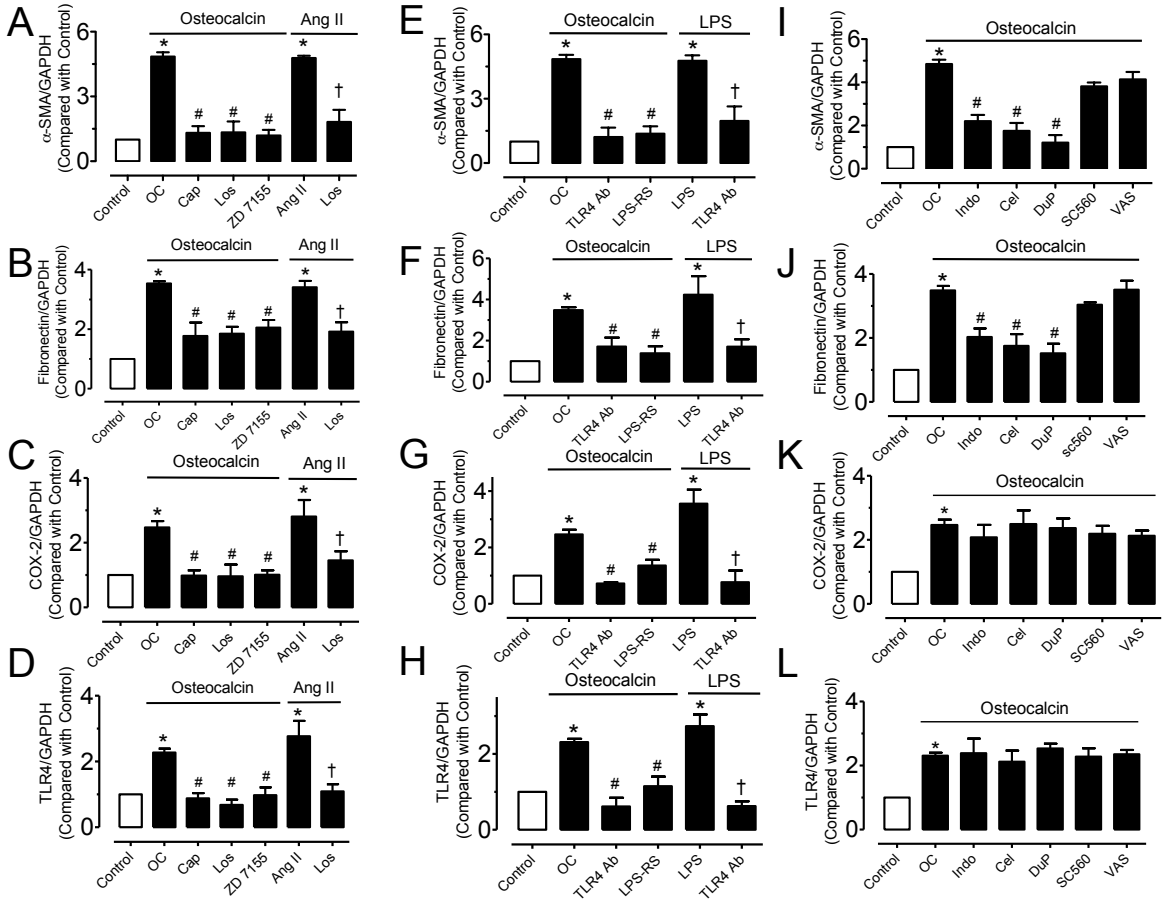


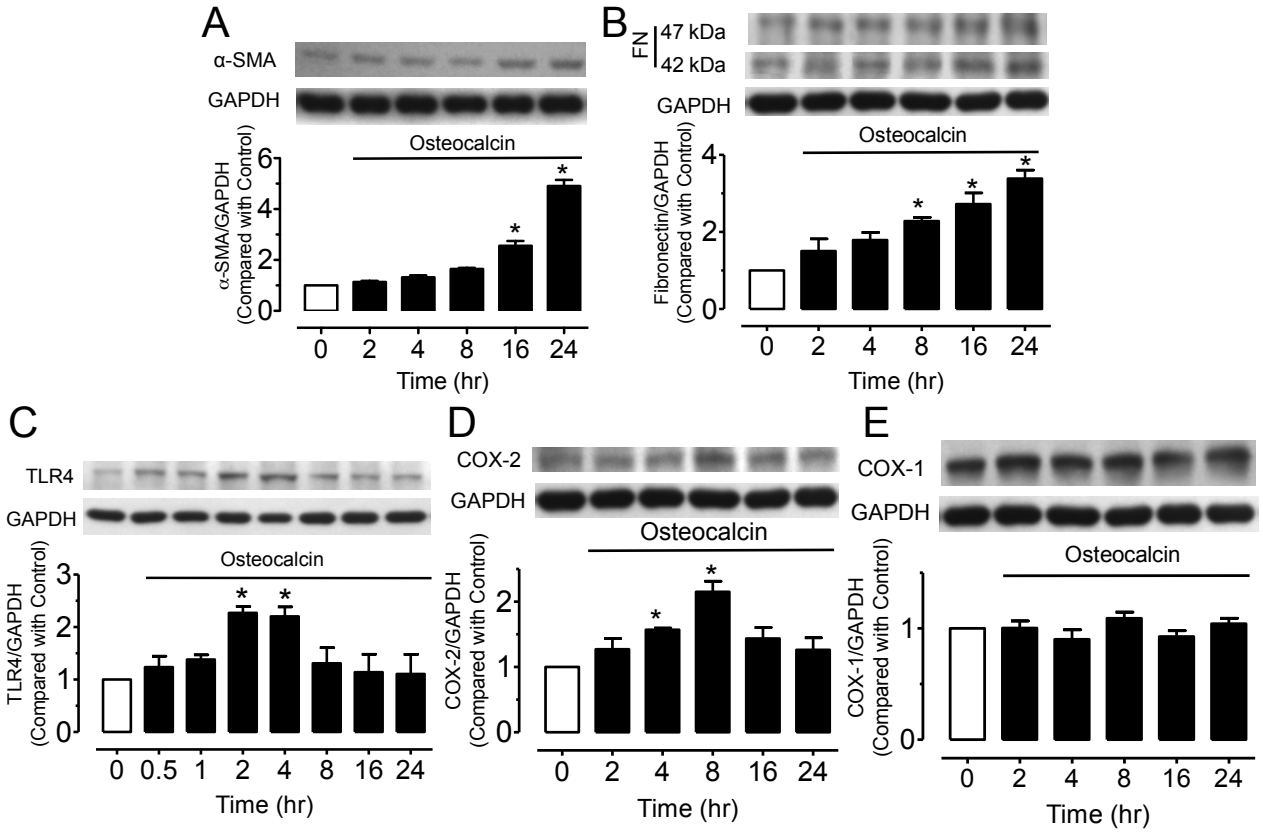
Supplementary figure I. Primary culture characterization using Western blotting and FACS. (A) Western blot on the markers for fibroblasts (prolyl 4-hydroxylase, P4H), smooth muscle cells (α-smooth muscle actin, α-SMA) and endothelial cells (platelet/endothelial cell adhesion molecule-1, PECAM-1). HUVEC, human umbilical vein endothelial cells; A_7r_5, rat smooth muscle cells. (B) FACS analysis on vimentin, P4H, α-SMA, PECAM-1 and myeloid cell marker CD33. SA, secondary antibody only (i.e. negative control).



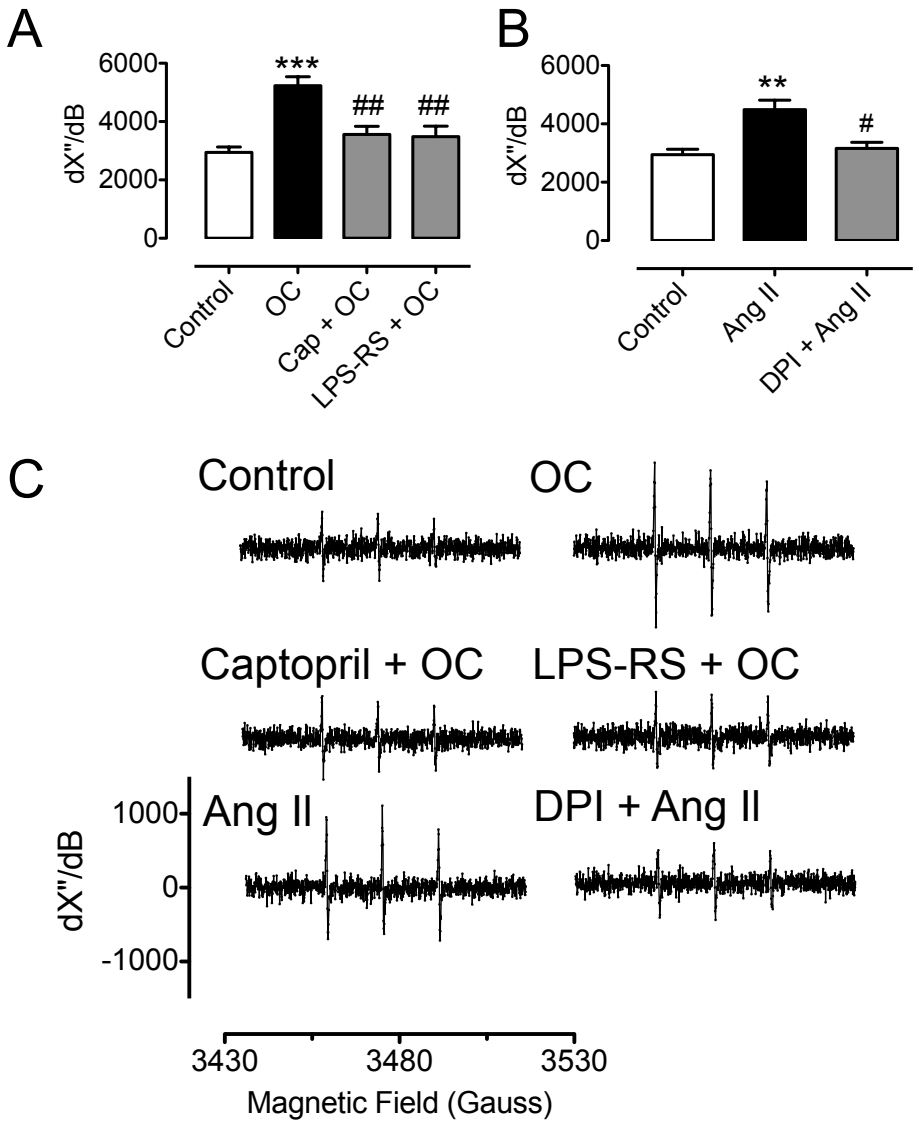
Supplementary figure II. Myofibroblast transformation in fibroblasts and adventitia and the expression of RAS in the fibroblasts. (A) Expression of α-SMA in osteocalcin (OC, 10 nmol/L, 24 hours)-treated fibroblasts. Scale bar denotes the length of 50 μm. (B) Expression of α-SMA and fibronectin (FN) in osteocalcin-treated ex vivo culture of perivascular adventitia. The expression of (C) angiotensin II type 1 receptor (AT_1R), angiotensin-converting enzyme (ACE) and (D) renin in the primary culture of rat aortic fibroblasts with or without osteocalcin treatment.



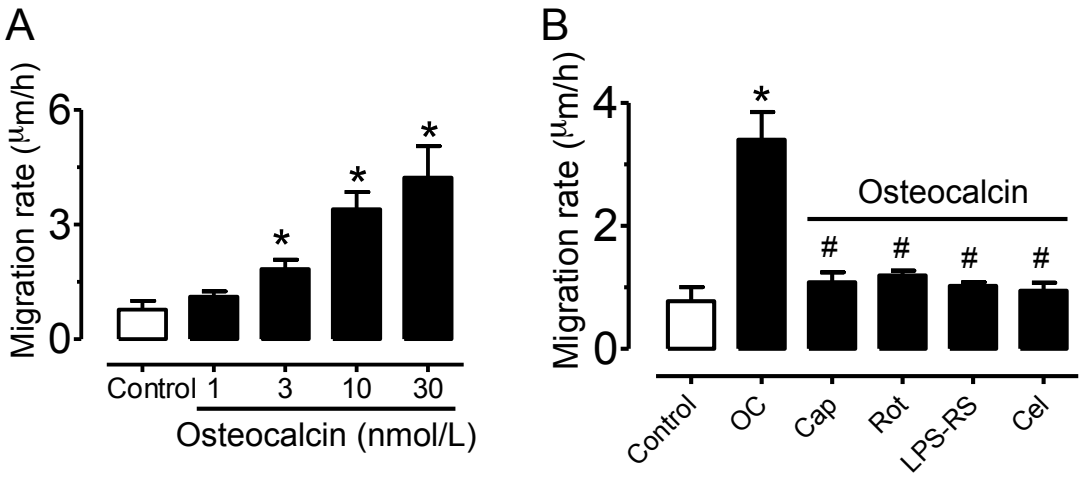
Supplementary figure III. Role of Ang II, TLR4 and COX-2 in osteocalcin-induced fibroblast transformation. Western blots showing the effect of (A-D) RAS inhibitors (Cap, captopril, ACE inhibitor, 100 nmol/L; Los, losartan, AT_1R antagonist, 3 μmol/L; ZD, ZD 7155, AT_1R antagonist, 3 μmol/L), (E-H) TLR4 neutralizing antibody (TLR4 Ab, 1 μg/mL), TLR4 antagonist (LPS-RS, 10 ng/mL) and (I-L) COX inhibitors (Indo, indomethacin, non-specific COX inhibitor, 3 μmol/L; Cel, celecoxib, COX-2 inhibitor, 3 μmol/L; DuP, DuP-697, COX-2 inhibitor, 3 μmol/L; SC 560, COX-1 inhibitor, 10 nmol/L; VAS, valeryl salicylate, COX-1 inhibitor, 30 μmol/L) in osteocalcin (10 nmol/L)-induced expression of α-SMA, FN, COX-2 and TLR4. One-way ANOVA; $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus OC; $^{\dagger}P<0.05$ versus Ang II or LPS.



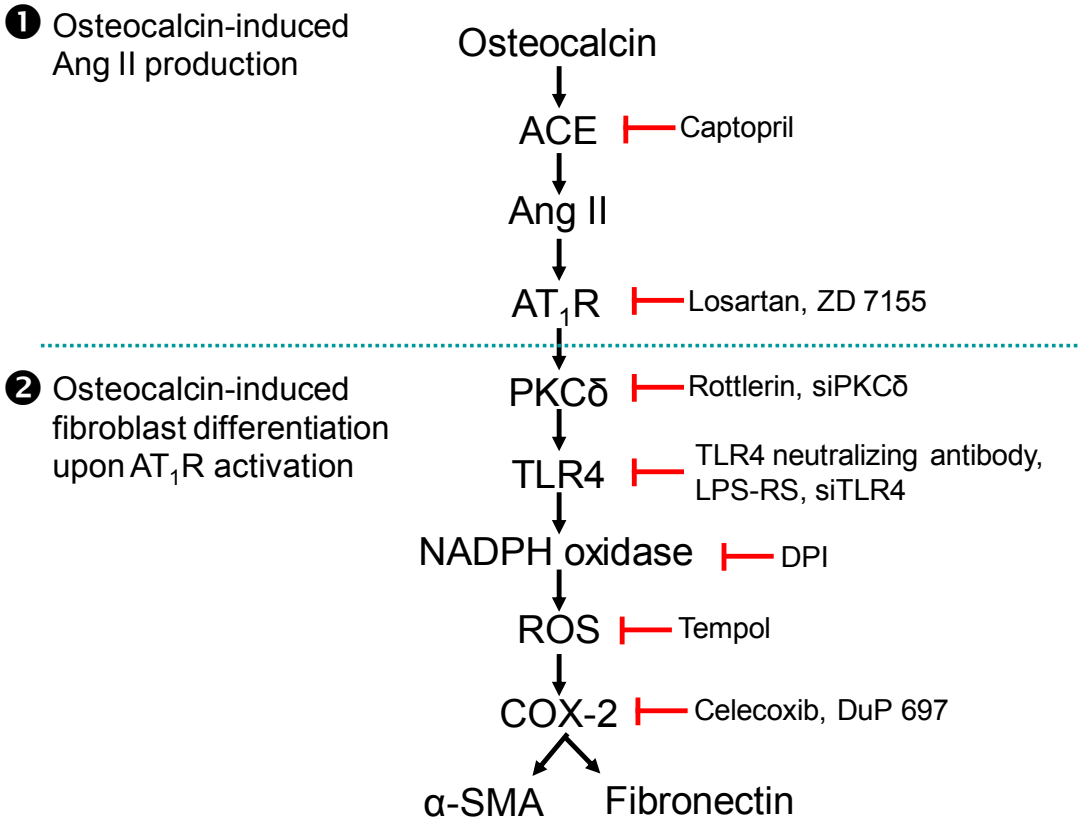
Supplementary figure IV. Time-dependent expression of α-SMA, FN, TLR4, COX-2 but not COX-1 by osteocalcin (10 nmol/L). Student's *t*-test; $^{*}P<0.05$ versus control (time at 0).



Supplementary figure V. EPR measurements of osteoclacin (10 nmol/L, 24 hours) or Ang II (100 nmol/L, 24 hours)-treated fibroblasts on ROS production. (A and C) Osteocalcin and (B and C) Ang II stimulated intracellular ROS production in the fibroblasts. ***P*<0.01 and ****P*<0.001 versus untreated control; $^{\#}P$<0.5 and $^{\#\#}P$<0.01 versus fibroblasts treated with osteocalcin or Ang II.



Supplementary figure VI. Concentration-dependent stimulation on fibroblast migration and the effect of various inhibitors on osteocalcin (OC, 10 nmol/L)-triggered migration. (A) Summarized data from wounding healing assay. Student's *t*-test, $^{*}P<0.05$ versus control. (B) Cap, captopril, ACE inhibitor, 100 nmol/L; Rot, rottlerin, PKCδ inhibitor, 10 μmol/L; LPS-RS, TLR4 antagonist, 10 ng/mL; Cel, celecoxib, COX-2 inhibitor, 3 μmol/L. One-way ANOVA; $^{*}P<0.05$ versus control; $^{\#}P<0.05$ versus OC.



Supplementary figure VII. Osteocalcin stimulates remodeling marker (α-SMA and fibronectin) expression by stimulating Ang II production which then activates the PKCδ/TLR4/ROS/COX-2 cascade. ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT_1R, angiotensin II type 1 receptor; PKCδ, protein kinase Cδ; TLR4, toll-like receptor-4; ROS, reactive oxygen species; COX-2, cyclooxygenase-2; α-SMA, α-smooth muscle actin.